

Tracing the Origin of the Black-spotted Frog, *Pelophylax nigromaculatus*, in the Xinjiang Uyghur Autonomous Region

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Abstract Identifying the origin of a biological invasion is critical for controlling the invaders. To explore the genetic diversity and identify the source region of introductions of *Pelophylax nigromaculatus* to the Xinjiang Uyghur Autonomous Region, we sequenced 695 bp of the mitochondrial Cyt *b* gene in 140 individuals of *P. nigromaculatus* and identified 42 haplotypes in Heilongjiang, Beijing, Jiangsu, Shaanxi, Zhejiang provinces, Chongqing and the Xinjiang Uyghur Autonomous Region. We detected only four mitochondrial haplotypes in 20 specimens from Yining city in Ili Kazak Autonomous Prefecture. We traced the origin of Yining *P. nigromaculatus* to the Beijing and Chongqing area. Our results extend the known distribution range of this species in Asia.

Keywords *Pelophylax nigromaculatus*, invasion genetics, Xinjiang, invasion route

1. Introduction

Biological invasions are a major threat to global biodiversity and have evoked concerns among evolutionary ecologists and biological conservationists (Pyšek *et al.*, 2008). Comparative studies that explore an invasive species in both its native and introduced ranges can reveal how an exotic species affects or is affected by a new environment (Meiners, 2007). Such studies not only establish the basic biological characteristics of an invader but also provide genetic information on the founding population of an invasive species, the invasion pathway of an exotic species throughout its introduced range, and its dispersal pattern (Campbell-Staton *et al.*, 2012; Ficetola *et al.*, 2008; Kolbe *et al.*, 2004; Peacock *et al.*, 2009). Therefore, these types of studies improve the prediction of the future distributions of invasive species.

When studying biological invasions, population genetic analyses based on molecular markers can provide valuable

information concerning population demographics, colonization history, and population structure (Ficetola *et al.*, 2008; Moule *et al.*, 2015; Rius *et al.*, 2015; Sherwin *et al.*, 2015). Moreover, this information can be used to detect the source populations for the original colonization and contemporary dispersal into the invaded range (Kolbe *et al.*, 2004; Peacock *et al.*, 2009). When developing management strategies, reconstructing the invasion history of invasive species can enhance our understanding of invasion risks by identifying the areas most susceptible to invasion and forecasting the future spread of such species based on past patterns of population expansion. Population genetics is a strong tool for enhancing the effectiveness and sustainability of management strategies (Liebl *et al.*, 2015).

The black-spotted frog (*Pelophylax nigromaculatus*) is widely distributed in China, far-eastern Russia, the Korean peninsula and Japan. In China, this species originated in the north, east, central and southwest mainland and eastern islands (Zhang *et al.*, 2008). Although there have been several (ecological, genetic and evolutionary) studies of the black-spotted frog in its native populations (Gao *et al.*, 2015a; Gao *et al.*, 2015b; Liu *et al.*, 2010; Wang *et al.*, 2014; Wang *et al.*, 2009;

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Wang *et al.*, 2008; Yang *et al.*, 2003; Zhang *et al.*, 2008; Zhang *et al.*, 2004), no invasion genetics surveys have been performed in China.

Ili Kazakh Autonomous Prefecture is located in the west of Xinjiang Uyghur Autonomous Region (79°50'30"–84°56'50" E, 42°14'16"–44°50'30" N). Yining is located on the northern side of the Ili River in Ili Kazakh Autonomous Prefecture. The Ili River valley is wetter than most of Xinjiang and contains rich rice fields. Yining has a semi-arid climate without strong variations of seasonal precipitation. In winter, the average temperature is –8.8°C. However because of the influence of the Dzungarian Altai and Boroboro Mountains to the northeast, the city is warmer than most of Xinjiang. Summers are hot with an average July temperature of 23.1°C. Species richness is lower in the Ili Kazakh Autonomous Prefecture than at similar latitudes elsewhere in China: 4 amphibian species (*Bufo viridis*, *Rana chensinensis*, *R. asiatica* and *Pelophylax ridibundus*) have been discovered in Ili Kazakh Autonomous Prefecture (Wang *et al.*, 2006).

The aim of the present study was to investigate population genetic patterns underlying the expansion of *P. nigromaculatus* in Xinjiang Uyghur Autonomous Region as inferred from mtDNA markers. We a) assessed the genetic diversity and examined the genetic structure of *P. nigromaculatus* in its native range in China and in the invaded territory of Xinjiang Uyghur Autonomous

Region; b) reconstructed the routes of species' expansion from its origin in East China to areas of recent colonization in West China; and c) discuss the application of these results to the planning of appropriate control measures.

2. Materials and Methods

Sampling of *P. nigromaculatus* In 2012, we collected black-spotted frogs from different locations in six native ranges: Qiqihaer, Beijing, Xuzhou, Xi'an, Wenzhou and Chongqing, because these locations encompass most of the distribution of this species in northeast, north, central, northwest, southeast and southwest China, and in 2014, we collected frogs from the region of introduction, Xinjiang Uyghur Autonomous Region (Figure 1). To determine whether a site has been invaded by *P. nigromaculatus*, we surveyed all accessible water bodies at each site for three consecutive nights with line transects. The third toe of the right hind-foot of each post-metamorphosed black-spotted frog was clipped, and the tissue samples were preserved separately in 95% ethanol and stored at –20°C in the laboratory.

DNA extraction and amplification DNA was extracted according to the procedure described by Wang *et al.* (2014) and Zhu *et al.* (2014), with some modifications. Briefly, we placed approximately 3 mg (wet mass) of

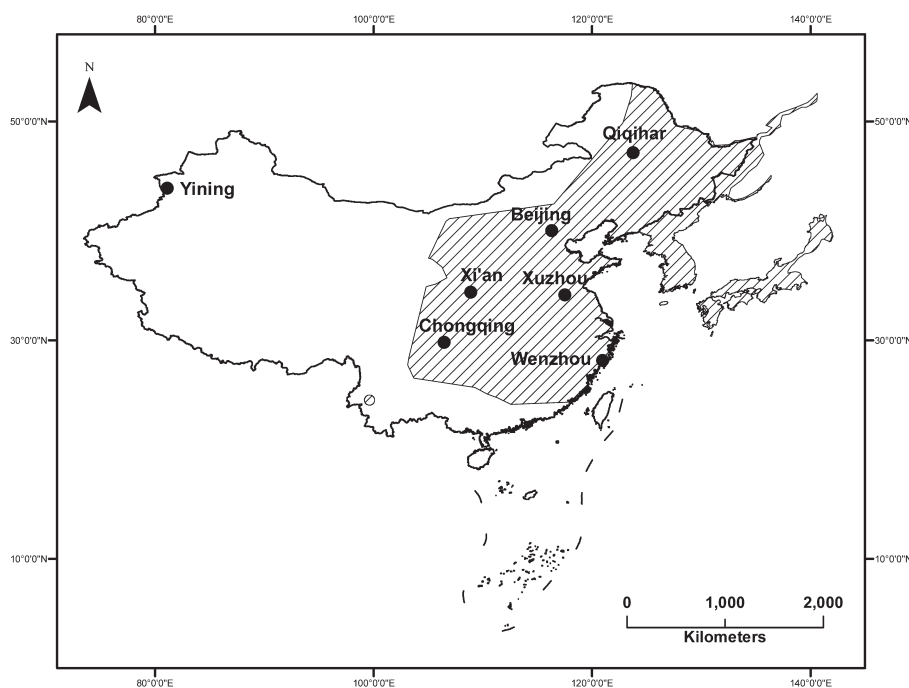


Figure 1 Sampled areas for *P. nigromaculatus* in China. Backward diagonal areas indicate distribution area in Asia. Closed circles denotes sampling site.

each sample in a 2 mL centrifuge tube with 100 μ L of lysis buffer containing 0.5% Nonidet P-40, 0.1 M EDTA, 0.01 M Tris-HCl (pH 8.0), 0.01 M NaCl, and 1 mg/mL proteinase K. The microtube was then shaken for 1 min using a vortex mixer at 16°C, followed by centrifugation for 1 min to concentrate all of the tissue at the bottom of the tube. Subsequently, the tube was incubated at 55°C for 24 h and at 100°C for 20 min. Finally, the microtube was centrifuged at 12,000 rpm for 3 min at 4°C, and the supernatant containing the DNA was diluted to one tenth of its original concentration for amplification via polymerase chain reaction (PCR).

We amplified a 695-bp segment of the mitochondrial cytochrome *b* (*cyt b*) gene from all specimens using the primers RanaLeuF5d (5'-AATMCCGWAAATCTCACCCCT-3') and RanacytbB1 (5'-GCTGGTGTAAATTGTCTGGGTC-3') (Yang *et al.*, 2003). The PCR products were then subjected to electrophoresis on 2% agarose gels. The resulting PCR products were directly sequenced using the same primers used for amplification (Beijing Genomics Institute, Beijing, China).

Data analysis The mitochondrial *cyt b* gene sequences were aligned and edited using MEGA 6 (Tamura *et al.*, 2013). The sequences were aligned using Clustal X, and unique haplotypes were identified using DnaSP 5.10 (Rozas *et al.*, 2003). The number of haplotypes (H_n), haplotype diversity (H_d) and nucleotide diversity (θ_π) within each sampling population were estimated by ARLEQUIN ver3.5 (Excoffier and Lischer, 2010). We employed TCS 1.21 (Clement *et al.*, 2000) to construct networks via statistical parsimony.

3. Results

H_n , H_d and θ_π ranged from 4 to 13, 0.363 to 0.932 and 0.00218 to 0.01731, respectively, among the sampled populations (Table 1). We also identified 42 haplotypes (H1–42), as defined by 116 polymorphic sites in the 695-bp *cyt b* fragment, among 140 *P. nigromaculatus*

specimens from seven populations in China. Of these, 35 were unique haplotypes, and seven (H1, H2, H4, H5, H6, H7 and H13) were shared among local populations (Table 2). The statistical parsimony network showed that the H1 and H4 haplotypes occurred in the Chongqing population, while the H2 and H4 haplotypes occurred in the Beijing population, suggesting that the *P. nigromaculatus* found in Xinjiang most likely originated from Chongqing and Beijing (Figure 2).

4. Discussion

Our study is the first to examine the invasion genetics of *P. nigromaculatus* in Ili Kazak Autonomous Prefecture in the Xinjiang Uyghur Autonomous Region. *P. nigromaculatus* is widely distributed in north, east, central and southwest China. However, *P. nigromaculatus* has only been noted within the area around Tcheng city of Xinjiang Uyghur Autonomous Region in the latest published records from northwest China (Jia *et al.*, 1993). Therefore, the new record from Ili Kazak Autonomous Prefecture extends the known distribution range of this species in Asia by approximately 344 km into Tcheng city (Jia *et al.*, 1993).

Historical records for the presence of *P. nigromaculatus* in Ili Kazak Autonomous Prefecture are not available, and therefore the pathways for invasion are unclear. The Shilei investigation report did not find *P. nigromaculatus* in the Xinjiang Uyghur Autonomous Region in 2006 (Shi *et al.*, 2007), indicating that the black-spotted frog likely arrived subsequently and established its population.

Our mtDNA data demonstrate that the recently established populations of *P. nigromaculatus* have reduced genetic variability compared to most native China populations (Table 1). However, the haplotype diversity in the two native populations (XA and CQ) was identical to that in the XJ invasive population (four haplotypes in the native population vs. four haplotypes in the invasive population). Two of the four haplotypes were shared with native Chinese samples from CQ populations, and one of

Table 1 Sampling information and mtDNA diversity of *P. nigromaculatus*.

Location	Abbreviation	N	H_n	$H_d \pm SD$	$\theta_\pi \pm SE$
Xinjiang	XJ	20	4	0.363 \pm 0.131	0.00281 \pm 0.00105
Xi'an	XA	20	4	0.616 \pm 0.067	0.00105 \pm 0.00019
Xuzhou	XZ	20	13	0.932 \pm 0.042	0.00818 \pm 0.00102
Beijing	BJ	20	11	0.895 \pm 0.052	0.00726 \pm 0.00141
Qiqihar	QQ	20	8	0.774 \pm 0.071	0.00218 \pm 0.00067
Wenzhou	WZ	20	6	0.579 \pm 0.124	0.00536 \pm 0.00162
Chongqing	CQ	20	4	0.742 \pm 0.051	0.01731 \pm 0.00146

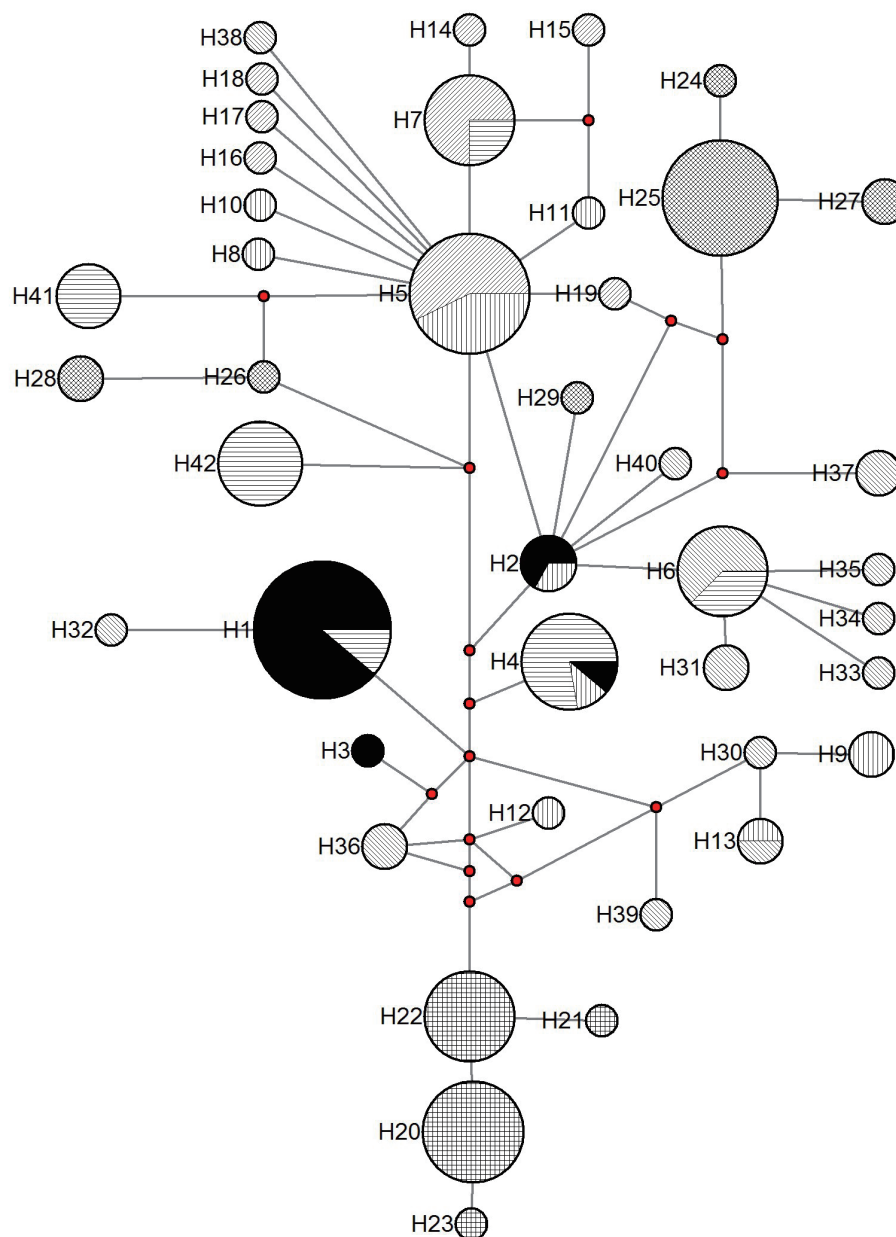


Figure 2 mtDNA haplotype parsimony network of *P. nigromaculatus*. Haplotype circle size is proportional to the number of individuals, numbers correspond to the haplotype numbers in Table 2. Solid represent Xinjiang population. Backward diagonal represent Qiqihar. Forward diagonal represent Xuzhou. Cross represent Xi'an. Diagonal cross represent Wenzhou. Horizontal represent Beijing. Vertical represent Chongqing.

the four was shared with the BJ population. These results suggest that the Beijing and Chongqing areas in China are likely the source regions of *P. nigromaculatus* in Ili Kazak Autonomous Prefecture. The escape of alien animals from markets represents a significant source of introductions for alien animals (Hulme *et al.*, 2008). Thus, the reason that these two areas are likely the source regions of *P. nigromaculatus* in Ili Kazak Autonomous Prefecture could be that they are both source areas for trade.

Our mtDNA analysis of *P. nigromaculatus* revealed

a clear genetic structure in native China (Figure 2). We detected few haplotypes that were shared among populations and significant genetic isolation in populations of China such as QQ, BJ, XA, XZ, WZ and CQ. These results indicate limited gene flow or migration among populations and are consistent with the results of our previous analysis of microsatellites (Wang *et al.*, 2014). Furthermore, we observed high genetic diversity in the central populations but low diversity in the marginal populations. This discovery is consistent with studies

Table 2 Population distribution of mtDNA haplotypes of *P. nigromaculatus*.

	XJ	XA	XZ	BJ	QQ	WZ	CQ
H1	16						2
H2	2			1			
H3	1						
H4	1			1			7
H5				6	8		
H6			5	3			
H7				2	6		
H8				1			
H9				2			
H10				1			
H11				1			
H12				1			
H13			1	1			
H14					1		
H15					1		
H16					1		
H17					1		
H18					1		
H19					1		
H20		10					
H21		1					
H22		8					
H23		1					
H24						1	
H25						13	
H26						1	
H27						2	
H28						2	
H29						1	
H30			1				
H31			2				
H32			1				
H33			1				
H34			1				
H35			1				
H36			2				
H37			2				
H38			1				
H39			1				
H40			1				
H41							4
H42							7

of the molecular phylogeography of *P. nigromaculatus* (Yang *et al.*, 2003; Zhang *et al.*, 2008).

Monitoring the variations of the genetic diversity of these recently introduced populations is important for analyzing invasion genetics because the variability will

increase over time as additional introduction events occur (Kolbe *et al.*, 2004). Intraspecific hybridization of individuals from genetically different populations (e.g., from Beijing and Chongqing) may increase the fitness (e.g., higher fecundity) of *P. nigromaculatus* populations in Ili Kazak Autonomous Prefecture. Furthermore, mixed populations might be better adapted to new environments (Fitzpatrick and Shaffer, 2007), which could lead to a variety of serious problems associated with *P. nigromaculatus* in Ili Kazak Autonomous Prefecture. Identifying the origin of invasive *P. nigromaculatus* populations could facilitate the exploration of migratory pathways and prevent the potential influx of other haplotypes that would increase the genetic diversity of this species.

Invasive taxa threaten biodiversity by replacing native biota (Fitzpatrick *et al.*, 2010; Rius and Darling, 2014). Reproductive interference (such as hybridization) is an important mechanism behind such replacement processes. Hybridization can have negative consequences (for example, the loss of locally adapted alleles, outbreeding depression and displacement by gene pool swamping) for the native population (Rius and Darling, 2014). Ili Kazak Autonomous Prefecture is home to another *Pelophylax* species (*Pelophylax ridibunda*). The results of the first interactions between *P. nigromaculatus* and *P. ridibunda* will provide important insights on amphibian specialization.

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